MICRONUCLEUS ASSAY IN UROTHELIAL CELLS IN CANCER CERVIX

This dissertation is submitted to

The Tamilnadu Dr.MGR Medical University, Chennai
in Partial Fulfillment of the Regulations for
D.M. Branch VII (Medical Oncology)
Madras Medical College, Chennai – 600 003.
August 2010

CERTIFICATE

This is to certify that this dissertation entitled "**MICRONUCLEUS ASSAY IN UROTHELIAL CELLS IN CANCER CERVIX"** is a bonafide record of original work done by **Dr. S. SURESH KUMAR**, under our guidance and supervision in the Department of Medical oncology, Madras Medical College, Chennai-600 003 , during the period of his Higher specialty study for DM(Branch VII) Medical Oncology from July 2007- August 2010.

DEAN
MADRAS MEDICAL COLLEGE
CHENNAI-600 003.

CERTIFICATE

This is to certify that this dissertation entitled "**MICRONUCLEUS ASSAY IN UROTHELIAL CELLS IN CANCER CERVIX"** is a bonafide record of original work done by **Dr. S. SURESH KUMAR,** under our guidance and supervision in the Department of Medical oncology, Madras Medical College, Chennai-600 003 , during the period of his Higher specialty study for DM (Branch VII) Medical Oncology from July 2007-August 2010.

Dr.K. VIJAYA SARATHY,MD.DM (MEDICAL ONCOLOGY)
PROFESSOR AND HEAD
DEPARTMENT OF MEDICAL ONCOLOGY
MADRAS MEDICAL COLLEGE
CHENNAI-600 003.

ACKNOWLEDGEMENT

It is with great pleasure, I acknowledge my deep and sincere gratitude to my beloved teacher Prof.K.Vijaya sarathy, Head of the Department of Medical Oncology,for his guidance ,constant encouragement and patience throughout the period of the study and my post graduate career.

I am greatly indebted to my co-guide Dr.Kanchana, Professor of Pathology,IOG,Egmore for extending her invaluable help for my study.

I express my sincere thanks to Dr.Radhabhai Prabhu, Director in Charge, IOG, Egmore.

I am thankful to Dr.Lakshmi Narasimhan and Dr.Balaji for their constant encouragement and support.

I acknowledge with thanks for the warm help extended to me in times of need by my colleagues and friends.

I owe my thanks to the technical staff of the Department of Pathology for their kind cooperation.

I express my gratitude to the Dean, Madras Medical College for permitting me to make use of the clinical facilities in the Institute.

With deep gratitude, I must thank all the patients without whose cooperation; this study would not have been possible.

Its my privilege to thank the Almighty and my family members for their blessings.

At the end it would be unjustified if I miss the name of my beloved wife Juju and my cute son Jujan whose constant encouragement and sacrifice in family matter enable to me to complete this work.

Suresh Kumar.S

CONTENTS

ABBREVIATIONS

HUMN	–	HUMAN MICRONUCLEUS
MN	–	MICRONUCLEUS
SES	–	SOCIOECONOMIC STATUS

INTRODUCTION

INTRODUCTION

Cancer, modern epidemics of non-communicable diseases is the second commonest cause of mortality in developed countries and remains one of the ten commonest causes of mortality in developing countries like India is a complex disease with altered expression, abnormal growth and disruption of normal function of cells caused by genotoxic effects resulting genomic instability at an early stage of cancer.

To evaluate the genotoxic risks/ effects, observed as DNA damages, can be assessed by chromosomal aberrations, sister chromatid exchanges and micronucleus test. Out of all these, micronucleus test is found to be the most sensitive when compared with other tests as it neither requires tedious procedures like cell culture and metaphase preparation, nor it requires any specific DNA stains. To further add, as it is applicable on interphase cell only , it is the best indicator of mitotic interference and chromosomal mutations or breakages and is noninvasive and economical too.

Micronucleus, a microscopically visible round or oval cytoplasmic chromatin mass in the extra nuclear vicinity, originates from aberrant mitosis. It consists of eccentric chromosomes, chromatid fragments or whole chromosomes which failed to reach spindle poles during mitosis. Micronuclei have been used as biomarkers for assessment of DNA damages. Micronuclei provide a measure of both, chromosome breakage and chromosome loss.

2

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

1. To identify the occurrence of micronuclei in normal and cancer cervix.
2. To identify the occurrence of micronuclei in risk factors of cancer cervix.
3. To identify the occurrence of micronuclei in different stages of malignancy.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Carcinoma cervix worldwide accounts for 15% of all cancer diagnosed in women [1]. It is the second most common cancer in women globally and 80% of these occur in developing countries. It occupies either top rank or second among cancers in developing countries, whereas, in the affluent countries does not find place even in the top five leading cancers.

In India it has been reported to be the commonest malignancy in women, comprising 24% of all cancers in female[2]. Almost 20 per 100,000 Indian women have cancer cervix and it has been estimated that one in 63 likely to suffer from it in her life time [3,4]. It is estimated that approximately 100,000 women develop cancer cervix every year. [5]

The Indian Council of Medical Research initiated a network of cancer registries under the National Cancer Registry Program (NCRP) in 1981 and data collection commenced in these registries from January 1982 [6]. Cancer incidence is generally expressed as Age Adjusted or Age Standardized Incidence Rates (AAR) per 100,000 persons according to world standard population. Among females, the most common sites of cancers are breast and cervix.

Cancer of the cervix has been the most important cancer in women in India over the past two decades. In older population based cancer registries (PBCR) Barshi and Chennai PBCRs have always recorded the highest incidence of cervix cancer [7]. Based on the data of the PBCRs, the estimated number of new cancers during 2007 in India was 90, 7086.

Cancer of the cervix accounted for 16 per cent of all cancers in women in the urban registries in 2005. Since over 70 per cent of the Indian population resides in the rural areas, cancer cervix still constitutes the number one cancer in either sex [6].

In all developing countries as in India, maternal morbidity and mortality are points of cynosure. However besides direct gynaecologic and obstetric complications, another important issue leading to these outcomes is the high incidence of cervix cancer especially among the economically – disadvantaged [15]

In fact the total incidence of mortality from cancer at all sites is greater in the lower socio-economic groups and is due mainly to increased incidence and mortality at certain sites including the cervix uteri [8].

Risk for cervical cancer has been reported to be variable from one population group to another because of its multifactorial etiology [9].

It appears that a larger proportion of the Indian female population is more vulnerable to cervical neoplasia, since the recognised risk factors for cancer cervix like illiteracy, low socio-economic status, early menarche, early marriage, multiparity, first child birth at an early age, poor genital hygiene and genital infections are widely prevalent in this population [3, 4].

Increasing age, increasing parity (Para > 3) age at marriage, clinical lesions of the cervix, gynecological complaints, STD were the risk factors for SIL and cancer cervix. 67.8% of the total SIL and 85.4% of the carcinoma cervix was observed in women with high parity (3 or more children). 51.5% of the total SIL and 75.3% of the total cancer cases were observed in women more than 40 years of age [6]

The highest age specific incidence rate for cancer was seen in more than 60 years of age. Cancer cervix patients were in the age group of 28 to 75 years and they had mostly married young in the age of 15 years [5].

The frequency of cancer cervix showed a progressive risk with increasing parity mostly between Para 2 and 3. [10]

The relative risk of cervical cancer increases with the more number of abortions [11].

There is a correlation between the low SES and cancer cervix. It has in fact been observed that uneducated women due to lack of knowledge of proper hygiene and preventive measures are more prone to cancer cervix [8].

However, cancer of the cervix responds favorably to secondary prevention measures as it has a long pre-clinical phase that usually requires 2 to 10 years to penetrate the basement membrane and invade tissues [12].

Statistics reveal that about 4 cases of every 5 cervical carcinoma patients actually occur in those countries that are without screening programs [13].

In fact in developed countries, 80% of cases are curable because of early detection while in developing countries, 80% of cases are incurable at the time of detection if they are detected at all [14].

Rather, women do not come forward for routine gynaecological examination due to lack of knowledge about its early symptoms, fear of cancer (fatalistic attitude) and lack of awareness about the possibility of a cure [15].

In a WHO bulletin in 2001 there were the recommendations regarding Effective screening programs in the developing countries like India for cervical cancer in low and middle income groups [16]

The choice of screening test in countries/ regions that plan to initiate new programs should be based on the comparative performance characteristics of cytology and its potential alternatives such as VIA (visual inspection with acetic acid) [17]

Since programs cannot afford the luxury of frequently repeated testing of women, a highly sensitive test should be provided. Owing to their limited resources, developing countries cannot afford the models of frequently repeated screening of women over wide age ranges that are used in developing countries [16].

If cytology is chosen, considerable attention should be given to obtaining good Quality smears, staining, and reporting so that a moderately high sensitivity to detect lesions is ensured. If a

potential alternative to cytology, such as VIA, is chosen for screening, considerable attention should be given to the proper monitoring and evaluation of the program inputs and outcomes before further expansion[16].

A single life time screening which appears to most affordable and feasible method of control of cancer cervix in developing countries like India should be carried out in all women of high parity (3 or more children) irrespective of age and in all women above the age of forty irrespective of parity was the conclusion of the study[18].

Regular screening can be achieved by imparting appropriate education to the masses regarding the early signs and symptoms of cervix cancer and utilizing rapid screening/ diagnostic measures.

Routine protocol empathizes that every female above 25 years should annually get a Pap smear examination as a screening procedure [15].

Marked reductions in morbidity and mortality from cervix cancer have been achieved among screened persons [15].

Mass screening by cytology, to detect precursors of cancer of uterine cervix, is among the most successful of health measures. Survival of cervix cancer patients is most directly related to the stage of disease at diagnosis, and the best way to increase detection of cervical neoplasm in pre-cancerous or localized stage is to improve the scope and quality of cytology screening [16].

Pap smear is the most frequently used test in mass screening programs introduced by George Papanicolaou in 1940, yet it is not totally reliable. [42]

The sensitivity of Pap smear for the detection for cervical cancer precursors is less than 50%. The rate of false negativity is about 20 to 30 % in women with high grade CIN and 10 to 15 % in women with invasive cancer [43]

Long term exposure to genotoxic factors leading to mutations has been proved to be related to the increasing incidence of carcinoma due to toxicity resulting in genomic instability being found in its early stages of carcinoma [21, 22].

The occurrence of genetic instability, either as a result of, or leading to, an increase in chromosomal rearrangements in most cancer types merits cytogenetic investigations.

The genomic instabilities have been observed in many forms like chromosomal instability[23], chromosomal breakage[21], nucleoplasmic bridges[23], micronucleus formation and double DNA strand breaks[22].Chromosomal instability usually results either from abnormal centriole formation or chromosomal loss in anaphase or mal-segregation of chromosome or mitotic slippage or failure of cytokinesis or nucleoplasmic bridges[23], whereas chromosomal breaks are caused by spindle apparatus and clastogenic mutations[21].

Among all these genomic instabilities seen to carcinomas, MN formation is the hallmark of genomic instability being defined as chromatin containing body that represents fragments or even whole chromosomes which failed to get incorporated into the daughter cell nucleus during mitosis or might result from DNA strand breakage[22].

The clastogenic effects can be also used as an effective screening measure for detection of cervical cancer in its pre-

clinical stage, in combination with morphological, biochemical and cytogenetic parameters, the Comet Assay along with the Micronucleus Test (MNT) may serve as novel tools to detect and predict the stage of cervical dysplasia [24].

Micronuclei can be detected in exfoliated cells of the buccal mucosa, urinary bladder, cervix or bronchi and seem to reflect chromatid and chromosome aberrations which occurred in the proliferating basal layers [23, 24, 25].

The sampling of such exfoliated cells is generally fast and highly economical; the MN assay has been the method of choice for survey of large population groups especially as a preliminary indicator for pre-cancerous lesions [23].

Since more than a century ago, micronuclei have been described by many scientists. In the late 1800s and early 1900s Howell and Jolly described Feulgen-positive nuclear bodies in human reticulocytes, known as Howell–Jolly bodies, and representing chromosomes separated from the mitotic spindle[26].

In the early 1970s the term Micronucleus test (MN Test) was suggested for the first time by Boller and Schmidt and Heddle who showed that this assay provided a simple method to detect the

genotoxic potential of mutagens after *in vivo* exposure of animals using bone marrow erythrocytes [26].

Many methods like chromosomal aberration analysis, sister chromatid exchange analysis and MN frequency have been indentified for detecting mutagen exposures [21]. Out of these MN assay has been found to be an important genotoxic screening test for the detection of agents which cause chromosomal damage and induced the formation of MN in interphase cells [22].

Chromosomal biomarkers of genomic instability related to carcinoma have been identified as MN formation, nuclear budding and non disjunction in the year 2002 by Fenech. As per his observations, the DNA damage rates in human population can best be measured by MN scoring rather than chromosomal aberrations [23]. The MN frequency was used in number of studies and was found to be of significant importance [27].

MN index has also been labeled as endogenous dosimeter in tissues that might be the future site for the development of carcinoma [28].

As observed, more than 90% of human cancers were epithelial in origin and exposure of dividing cells to mutagens

resulted either in chromosomal breakage or in MN formation, so MN assay can be used to assess the genetic damage in such tissues [29]

The cells from the basal layer of epithelium continuously divide differentiate and migrate to the upper cell layers. The MN frequency was observed from these exfoliated epithelial cells. Induction of MN, in processes associated with DNA damages like cancer, aging and other genetic disorders, has been considered as an effective biomarker [30].

Since more than 90% of the human cancers arise from the epithelial tissue, exfoliated cells hold a strong potential as a tool for biomonitoring human populations exposed to genotoxic agents. Various studies indicate the presence of MN in the exfoliated cells of buccal mucosa, nasal mucosa, urothelium, lymphocytes and erythrocytes[21].But, of all these tissues and cells, exfoliated cells have been considered as best surrogate for predicting cancer and they can be easily collected from the mouth, nose,and bladder by noninvasive procedures[30].

The micronucleus test is used as a biomarker for chromo-some instability and malignancy, observing higher frequencies of

micronucleated cells among cancer patients than among healthy individuals [31].

A gradual increase in MN frequency has been observed from normal to precancerous to cancerous lesions [32].

Many genotoxic studies on MN frequency were conducted related to predisposing factors which conclude significantly higher MN frequency in people with risk factors [33].

Number of micronuclei correlates with the severity of genetic damage. Cells containing several micronuclei present greater genetic damage than do cells that present only one micronucleus [34].

Human Micronucleus Project (HUMN) is an international collaborative study on the use of micronucleus technique for measuring DNA damage in humans. It validated the MN assays in human cells [30].

Epithelial tissues are in immediate contact with inhaled and ingested genotoxic agents, and kidney and bladder cells are also in contact with metabolites of the chemicals. Genotoxic changes in bronchial, esophageal, cervical, breast duct and other types of epithelia have also been reported [30].

An important component in the interpretation of MN assay results is cell kinetics. MN observed in exfoliated buccal, nasal or urothelial epithelia are not induced when the cells are at the epithelial surface, but when they are in the basal layer [39].

In general, cells take 7–16 days to emerge to the surface and exfoliate [30].

Variability of MN assessment arises from intraindividual variability, interindividual variability, and population variability, as well as variations characteristic of different cell types. When spontaneous MN frequencies were compared in different healthy individuals, up to a 17-fold difference was observed, possibly reflecting genetic and nonspecific exposure differences. HUMN[30].

The average reported healthy population MN frequency is 1–3 per 500 - 1000 cells, with no significant variation between different types of exfoliated cells. Repeated scoring of MN in epithelia from the same individuals showed variation between 30 and 102.9%.HUMN[40].

Increases in the MN frequency in exfoliated cells were also observed as a result of exposure to pesticides or neoplastic drugs

radiotherapy, smoking, arsenic in drinking water w56x, and chronic infection. Exposures to low levels of benzene or leather tanning solutions did not cause significant increases in cytogenetic damage[30].

A causal association between MN frequency and cancer risk could be inferred from studies of structural chromosomal aberrations(CAb) and aneuploidy. Somatic chromosome damage is involved in cancer etiology. Such damage can occur via chromosome loss as well as by breakageThere is a direct association between the frequency of MN in target or surrogate tissues and cancer development [30].

Michael Fenech have given detailed description of the scoring criteria for the micronucleus assay (MN ASSAY) in the HUMN project [41].

1. Morphologically identical but smaller than nuclei
2. Round or oval in shape
3. Diameter between 1/3rd and 1/16th of main nuclei
4. Nonrefractile
5. Not linked to main nucleus

6. May touch but not overlap the main nucleus and the micronuclear boundary should be distinguishable from the nuclear boundary.

7. Same color as nucleus

8. Similar staining intensity or more as nucleus

There are three mechanisms that may contribute towards the formation of micronuclei: metabolic stress caused by tumor growth, clastogenic products released from tumor cells and the presence of HPV.

Micronuclei is detected in the the exfoliated cells of the buccal mucosa, urinary baldder, cervix or bronchi and seem to reflect chromarid and chromosome aberrations which occurs in the proliferating basal layers[25].

The sampling of the exfoliated cells is fast and economical, the MN assay has been the method of choice for survey of large populations especially as a preliminary indicator for precancerous lesions[30].

The presence of micronuclei has been considered to be a very useful biomarker for detecting malignant cervical uterine carcinomas[35].

A significant MN count was observed in the presence of risk factors like illiteracy, low SES, early marriage and more number of conceptions[15].

A significant MN assay was observed in patients who were between 21 to 30 years and 51 to 60 years of age[36].

The maximum number of MN was seen in the age group between 40 to 60 years of age[34].

The frequency of MN cells increases with advancing age and that observed in 51 to 60 years age group are 3 folds higher than in age group of 21 to 30 years[36].

When the MN count in patients less than 17 years of age was compared to that of patients more than 18 years, non significant result was observed[37].

For the factor age at marriage significant MN count was seen in patients less than 17 years of age[36]

The trend for percent frequencies of MN cells at different parity levels exhibits non significant difference at low parity level but statistical difference at the higher parity levels[36,37].

There is a significant difference in MN count in the lower SES and middle SES group[36].

There is a strong association between MN frequency and staging of cancer cervix and there is a linear association both MN frequency and stage of the cancer cervix. The highest frequency of MN observed in stage III B[37].

There is an association between lesion severity and micronucleus frequency in epithelial cells, which contributes towards validating micronucleus frequency as a possible biomarker for cancer risk[38].

Significant MN count was observed even without abnormal PAP smear. This is because the sensitivity of MN assay in detecting the genomic instability at an early stage in the carcinogenesis[37].

The sensitivity and specificity for MN assay in urine cytology was72 % and 83.3% respectively. The efficiency was found to be 77.55%. This test has got a good negative predictive value[15].

Percent frequencies of micronucleated cells were highest in the patients with stage III, older patients, younger age at marriages, increased parity and low socioeconomic status[36].

The test in urothelial cells indicates damage in the tissue which is not site of cancer cervix. They also observed that elevated micronucleus frequencies parallel with the cytogenetic damage[36].

The frequency of MN in lymphocytes (0.18), buccal smears (2.40) and urothelial cells (0.5 to 1) in control groups is generally reported to be low[15].

Damage in urothelial cells was increased despite the lesser number of scorable cells and damage in cervix smears was also significantly more than in controls yet it was generally lesser than in urothelial cells except when analysed for the age variable. This is quite surprising since urothelial cells do not constitute cells of the cervix but comprise cells from the renal tubular, squamous and bladder epithelia[36].

The percent frequency of MN ranged from 0.101 to 0.756 while the cells scored varied from 0 to 9 in the patient population. The over all frequency of MN cells (0.324 ± 0.024) in the patients'

group was statistically significant (0.031 ± 0.023) from that observed in control (MN present in 24% of subjects) individuals. In the control group individuals, the highest frequency of MN observed was 0.220[15].

The frequency of MN cells in the buccal smears lymphocytes and urothelial cells has been observed to be low in the controls.Specifically when MN assay were made in the urothelial cells in the healthy controls it was observed to be low[36].

Percentage of MN among 10 cancer patients was 1.36%, in controls it was 0.35% only[15].

There was significant increase in the number of MN in cancer patients prior to the initiation of chemotherapy and radiotherapy when compared with healthy subjects[38].

MN can be used as a biomarker and as an important assay to find out the cancer in early stages. MN in exfoliated cells reflect genotoxic events that occurred in the dividing basal layer 1-3 weeks earlier. An assay in exfoliated cells holds promise as a site specific biomarker of exposure to genotoxins for cancer risk.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present descriptive study was undertaken in the patients attending the gynecology out patient department with complaints of leucorrhea, post-coital bleeding, lower abdominal pain, inter menstrual bleeding and prolongation of menstrual bleeding.

Patients were grouped in the following 2 categories:

1. Patients whose Visual Inspection of Cervix is normal were taken as Group A.
2. Patients whose Visual Inspection of Cervix shows positive findings were taken as Group B.

A Standard Performa was prepared in order to record the history, general examination and pelvic examination.

After making the proper recordings in the respective Performa, urine sample were collected from the patients.

After collection of the urine sample, the slides were prepared following the protocol given by Chakrabarti and Dutta et al (1988) for the MN assay.

Materials required:

1. Slides (microscopic).
2. Sterile plastic containers.
3. Cover slips.
4. Methanol.
5. Giemsa stain.
6. May-Grunwald's stain
7. Methanol
8. Phosphate buffer
9. Distilled water

PROCEDURE:

Collection of Specimens:

1. Patients
2. Local clinical examination were done by using Cuscos self retaining vaginal speculum and the different lesions were noted.

    - Erosion cervix – a bright red area surrounding and extending beyond the external os on the ectocervix with a clearly demarcated outer edge.
    - Hypertrophied cervix – the size of the cervix is enlarged.

- Suspicious and unhealthy cervix- if abnormal growth, ulcer or vasculature is seen.

3. Erosion and unhealthy cervix – cervical biopsy done and staged later if malignancy documented.

4. Obvious growth cervix was staged by clinical examination (FIGO staging system).

5. Patient was requested to collect mid stream urine sample (~ 10 ml) in sterile plastic containers. And were transported to the laboratory. They were processed within 3-4 hrs of sample collection. The procedure, briefly, entailed sample wash thrice in phosphate buffered saline (PBS) with alternate centrifugations at 1200 rpm for 10 minutes. From the pellet, smear preparations were made on pre-cleaned glass slides. Up to 2-3 slides per case were made and these were allowed to air dry. After air drying, the slides were kept absolute methanol for 20 minutes for fixation of cells.

Preparation of stains:

1. May-Grunwald's stock solution:

    - 25 mg of May-Grunwald powder was mixed with 100 ml of methanol
    - It was mixed properly with help of a mixer
    - It was filtered properly and stored in dark coloured air tight bottle.

2. Giemsa stock solution :

    - Giemsa powder 1 gm was dissolved in 60 ml of glycerin and kept in water bath at 60 * Celsius for 2 hrs.
    - Mixture was cooled to room temperature and 66 ml of absolute methanol was added, thus made solution was filtered and stored in dark airtight bottle.

Preparation of working solution

1. Giemsa working solution was prepared in the proportion of 3 parts of stock solution and 1 part of distilled water.

2. May –Grunwald working solution was prepared in the proportion of 2 parts of stock solution and 1 part of distilled water.

Procedure of staining:

- The slides were kept in May-Grunwald stain for 5 minutes.
- The slides were rinsed twice with distilled water.
- After washing , it was counter stained with Giemsa satin for

  8 to 10 minutes, followed by washing with distilled water.
- Stained slides were mounted with cover slip
- They were observed for nuclear abnormalities under bright field binocular microscope under low power (40 x).
- The presence of Micronucleus was confirmed under oil immersion (100 x).
- Observations were recorded and tabulated.

Method of Analysis:

Five Hundred cells were recorded in each patient from the slides prepared and the incidence of micronucleus were recorded and the collected data was subjected to Student's Independent't' test.

OBSERVATIONS

OBSERVATIONS

The present study was conducted from April 2009- May 2010. A total number of 60 patients who had undergone clinical examination for gynaecological complaints in the gynecological out patient dept at the Institute of obstetrics and Gynecology, Egmore were included in our study.

Observations were recorded on the personal history and clinical examination findings and the MN assay. The observed data was tabulated for analysis.

Out of the 60 cases, 23 cases had no findings on Visual inspection of cervix (GROUP A), 29 cases had growth cervix on examination and 8 cases had erosion cervix on examination. Both were taken as (GROUP B). **TABLE 1**

Out of the 60 cases, 23 cases in GROUP A were in the age group from 18 to 65 yrs and 37 in GROUP B were in the age group 21 to 72 yrs. **TABLE 1**

According to the patients age the patients were divided into 4 groups.

< 30 yrs group1,30–39yrs grp2,40– 49yrs grp3 and > 50 yrs grp 4.**TABLE 9**

The mean age in GROUP A was 36.5yrs and in GROUP B was 44yrs. **TABLE 2**

A statistically significant difference was observed between the two groups in terms of age. **TABLE 3**

Among the 60 cases 4 were commercial sex workers. The rest 56 cases, 12 GROUP A cases and 12 GROUP B cases got married before the age of 18 yrs.**TABLE 4**

Among the 56 cases, 26 GROUP B cases and 13 GROUP A cases had history of abortions (> 1) and in 10 GROUP B cases and 7 GROUP A cases there was no history of abortions. 17 cases had 1 abortion, 13 had 2 abortions and 9 had more than 3 abortions. **TABLE 5**

Among the 36 cases in GROUP B, 17 had more than 3 childbirths and 17 had 2 childbirths. Among the GROUP A, 6 had more than 3 childbirths and 14 had 2 childbirths **TABLE 6**

Among the 60 cases 17 were in the lower socioeconomic status and 26 were in the upper lower SES. Only 17 were in the middle SES.**TABLE 7**

Among the 37 cases in GROUP B , distribution of the cancer cervix cases were 1 in stage IA , 4 STAGE IB , 8 in STAGE IIA ,6 in STAGE IIB, 5 in STAGE III A & 6 in STAGE IIIB. **TABLE 8**

37% of the GROUP B cases who had significant MN counts were more than 50 yrs of age. But significant MN counts were seen in cases < 30 yrs and 40 to 49 yrs in GROUP A . This difference was statistically significant. **TABLE 9**

60% of GROUP A cases got married before the age of 17 and they had significant MN count. The rest 40 % of GROUP A cases got married after 18yrs had significant MN count. But in GROUP B cases significant MN count was equally distributed in both groups (50 % distributed equally). The difference was not statistically significant. **TABLE 10 .Chi square test 0.660**

50% of GROUP A with significant MN counts had 2 abortions and 55.6% of GROUP B with significant MN counts had 1 or 2 abortions. 35% of GROUP A cases and 27% of GROUP B cases with no history of abortions had significant MN counts. 15%

of GROUP A cases and 16% of GROUP B cases with 3 abortions had significant MN counts. But it was not statistically significant. **TABLE 11**

70% of GROUP A cases and 53% of GROUP B cases with less than 2 child birth had significant MN counts. 30% of both GROUP A and GROUP B cases who had 3 childbirth had significant MN counts. No cases in GROUP A with more than 3 children had significant MN count. 16% in GROUP B with more than 3 children had significant MN count. **TABLE 12**

22% of GROUP A cases and 32% of GROUP B cases in the lower SES had significant MN counts. 45% in GROUP A and 43% in GROUP B, in the upper lower SES had significant MN counts. 32% in GROUP A and 24% in GROUP B, in the middle SES had significant MN counts**. TABLE 13**. But the MN count in the upper SES was not statistically significant.

Among the GROUP B cases, 21.6% with cancer cervix stage IIA, 18.9% with cancer cervix stage III B had significant MN counts. Among the GROUP A cases significant MN counts were seen in 18.6%.The difference was proven to be statistically significant**. TABLE 14**

There is a linear association between mean MN count and stage of the cancer cervix. **TABLE 15**

The sensitivity of the MN test in GROUP A was 83.8 % and specificity was 82.6 % .The Efficiency of the test was found to be 83.3 %. **TABLE 16**

TABLES AND FIGURES

TABLES AND FIGURES

DISTRIBUTION OF CASES IN GROUP A & GROUP B

TABLE 1

VALID	FREQUENCY	PERCENT
1	23	38.4
2	29	48.3
3	8	13.3
TOTAL	60	100

GROUP A – 1 (VISUAL INSPECTION OF CERVIX – NORMAL)

GROUP B – 2 AND 3 (VISUAL INSPECTION OF CERVIX – ABNORMAL)

(2 = GROWTH CERVIX , 3 = EROSION CERVIX)

MEAN AGE IN GROUP A & GROUP B.

TABLE 2

Group	N	Mean	Std.deviation	Median	Minimum	Maximum
A	23	36.59	12.912	39.00	18	65
B	37	44.84	11.934	43.00	18	72
TOTAL	60	41.76	12.843	40.00	18	72

T TEST

Table 3

	T test for equality of means		
	t	**df**	**Sig (2-tailed)**
Age Equal variances assumed	-2.490	57	.016
Equal variances not assumed	-2.440	41.5	.019

Age is statistically significant

Distribution of cases according to Age at marriage

TABLE 4

Age in years	*Frequency*	**Percent**
12	1	1.7
13	2	3.4
14	4	6.8
15	10	16.9
16	9	15.3
17	4	6.8
18	12	20.3
19	8	13.6
20	1	1.7
21	1	1.7
22	2	3.4
23	0	0
24	1	1.7
25	1	1.7
CSW	4	6.8
TOTAL	60	100

Distribution of cases according to the Number of Abortions

Table 5

Number of Abortions	FREQUENCY	PERCENT
0	17	28.8
1	17	28.8
2	13	20.4
3	6	9.1
4	3	5.1
CSW	4	6.8
TOTAL	60	100

Distribution of cases according to Parity

Table6

Number of Parity	Frequency	Percent
1	3	5.1
2	30	50.8
3	17	28.1
4	5	7.5
5	1	1.7
CSW	4	6.8
TOTAL	60	100

Distribution of cases according to Socioeconomic status*

Table 7

Socioeconomic status*	FREQUENCY	PERCENT
1	17	28.3
2	26	43.3
3	13	21.6
4	4	6.8
TOTAL	60	100

** modified kuppuswamy scale.

1= LOWER SES , 2 = UPPER LOWER SES , 3 = LOWER MIDDLE SES , 4 =UPPER MIDDLE SES.

Distribution of cases according to Cancer cervix Stage.

Table 8

Stage	**FREQUENCY**	**PERCENT**
I A	1	3.4
I B	4	13.6
II A	8	27.2
II B	6	20.4
III A	5	17.0
III B	6	20.4
TOTAL	30	100

Frequency of MN count in different Age groups

Table 9

Group	Age (yrs)	MN count	
		0 – 3	**4 & more**
A	< 30	44.4%	25%
	30- 39	11.1%	0
	40- 49	38.9%	50%
	> 50	5.6%	25%
B	< 30	16.7%	9.7%
	30- 39	50%	22.6%
	40- 49	33.3%	29%
	> 50	0	38.7%

Frequency of MN count according to Age at marriage**

Table 10

Group	Age at marriage (yrs)	MN count	
		0 -3	4 & more
A	Less than 17	64.7%	33.3%
	More than 17	35.3%	66.7%
B	Less than 17	50%	50%
	More than 17	50%	50%

*** 4 commercial sex workers were not included in the analysis*

Frequency of MN count according to Number of Abortions

Table 11

Group	**No. of Abortions**	**MN count**	
		0-3	**4 & more**
A	Nil	41.2%	0
	1 – 2	52.9%	33.3%
	3 & more	5.9%	66.7%
B	Nil	66.7%	20%
	1 – 2	33.3%	60%
	3 & more	0	20%

Chi-Square Tests

Group		Value	df	Asymp. Sig. (2-sided)
A	Pearson Chi-Square	7.712[a]	2	.021
	Likelihood Ratio	6.588	2	.037
	Linear-by-Linear Association*	5.475	1	**.019***
	N of Valid Cases	20		
B	Pearson Chi-Square	5.760[b]	2	.056
	Likelihood Ratio	5.977	2	.050
	Linear-by-Linear Association*	5.000	1	**.025***
	N of Valid Cases	36		

THERE IS A LINEAR ASSOCIATION BETWEEN THE MN COUNT AND NUMBER OF ABORTIONS.

Frequency of MN count according to Parity.

Table 12

Group	Parity	MN count	
		0-3	4 & more
A	Less than 2	76.5%	33.3%
	3	23.5%	66.7%
	4 & more	0	0
B	Less than 2	83.3%	46.7%
	3	16.7%	60%
	4 & more	0	20%

frequency of MN count according to SES**.

TABLE 13.

Group	SES	MN count	
		0-3	4 & more
A	1	16.7%	50%
	2	50%	25%
	3	33.3%	25%
B	1	33.3%	32.3%
	2	50%	41.9%
	3	16.7%	25.8%

***Modified Kuppusamy scale.SES : 1 = LOWER , 2 = UPPER LOWER , 3 = LOWER & UPPER MIDDLE*
MN CELLS : 1 = 0 to 3 cells , 2 = 4 & more cells.

Frequency of MN count in Cancer cervix stage.

Table 14

CANCER CERVIX STAGE	MN frequency
I A	***2.9%***
I B	**11.4%**
II A	**22.9%**
II B	**17.1%**
III A	**14.3%**
III B	**17.1%**

Chi square test

	Value	***df***	***Asymp.Sig(2 sided)***
Pearson Chi Square	41.980	7	.000
Likelihood ratio	53.452	7	.000
No. valid cases	60		

Statistically significant

Data for MN assay in Urothelial cells in Group A & Group B

TABLE 16

MN assay in Urothelial cells	Test Results (no. of individuals)		Total
	Group A	Group B	
MN present	4	31	35
MN absent	19	6	25
Total	23	37	60

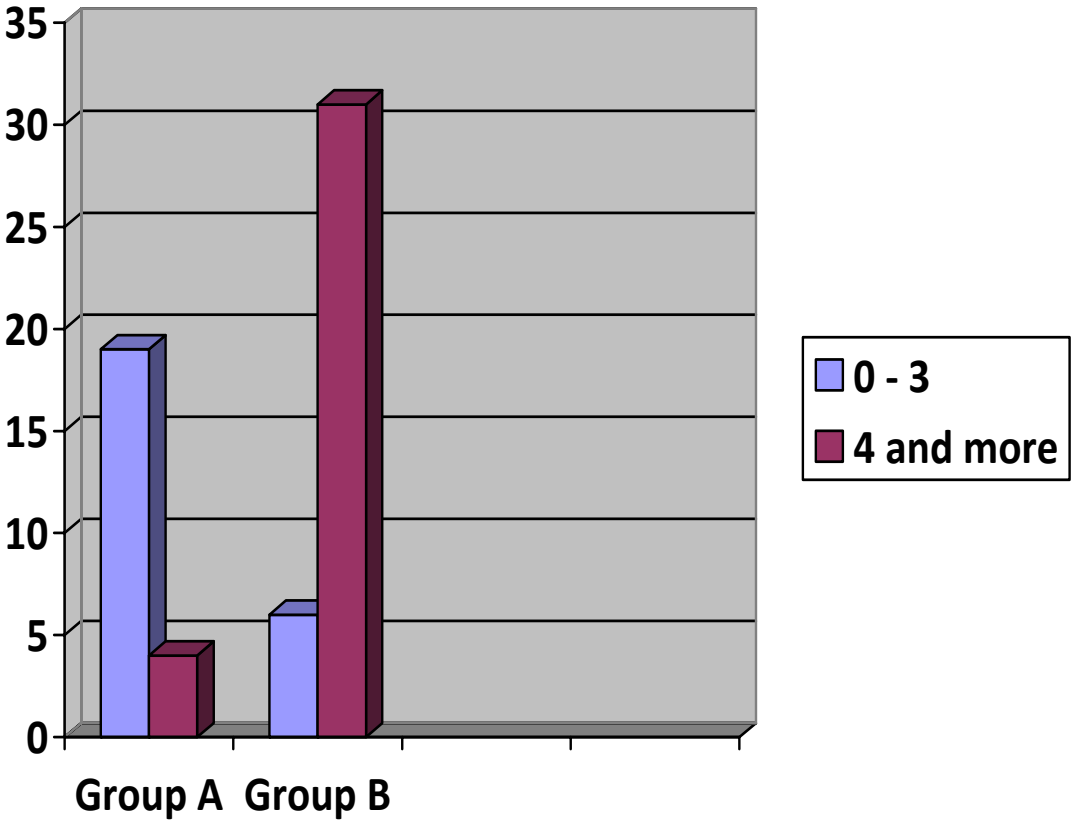
SENSITIVITY - 31/ 37= 83.8%

SPECIFICITY – 19 / 23 = 82.6 %

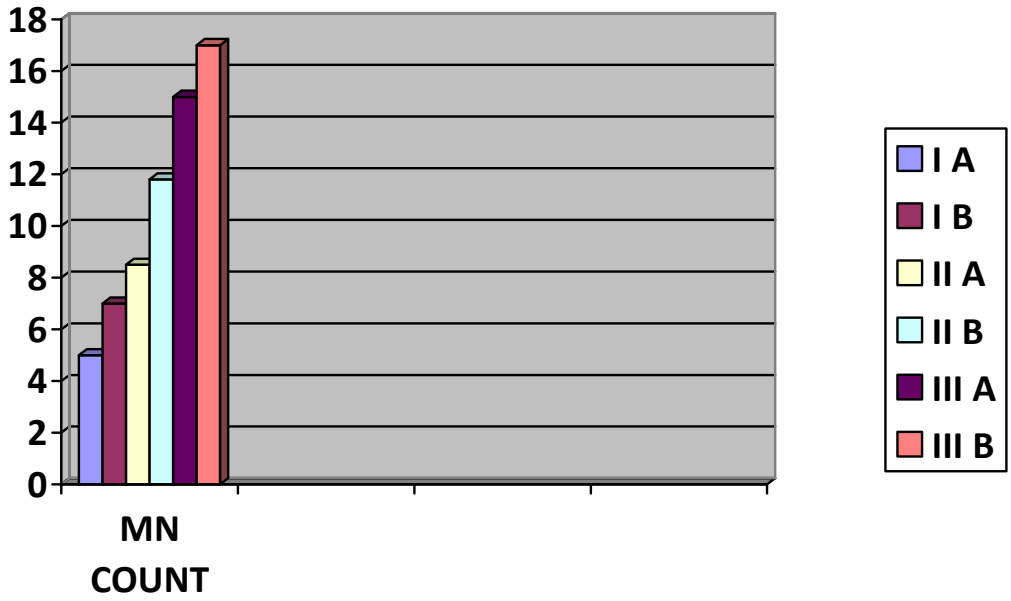
EFFICIENCY = 50 / 60 =0.833= 83.3%

DIAGRAMS AND CHARTS

- ***Data for MN assay in Urothelial cells in Group A & Group B***



Cancer cervix stage and Mean MN count.



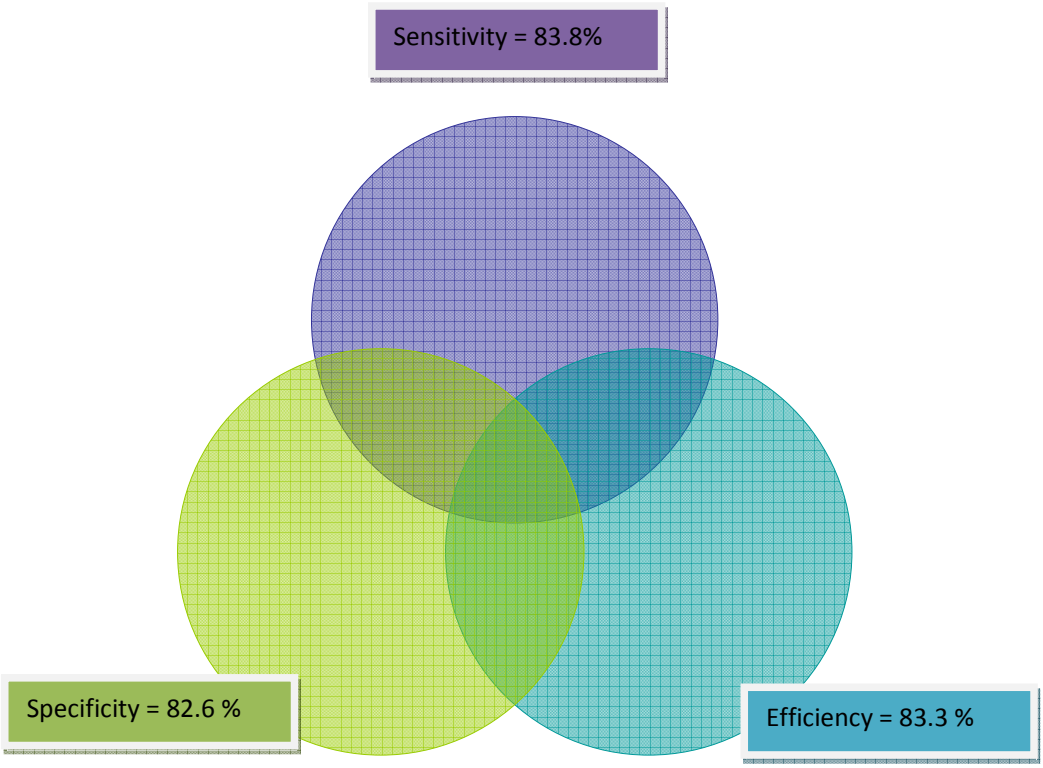
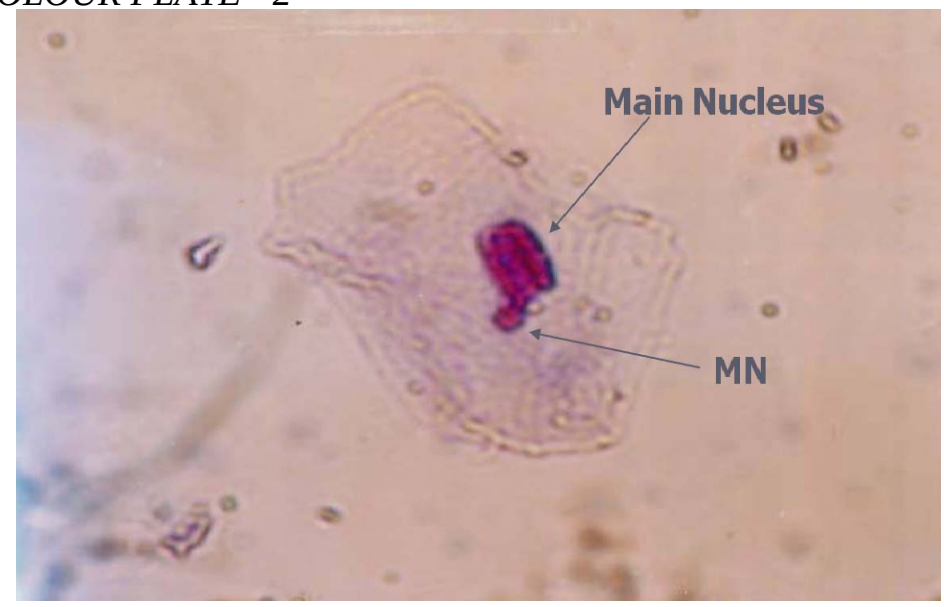


Diagram showing Sensitivity , Specificity & Efficiency of MN count in Group A cases.

COLOUR PLATE –1

Arrow showing micronucleus

COLOUR PLATE - 2



Arrow showing budding micronucleus

DISCUSSION

DISCUSSION

Complexity of the cancer could be attributed to its altered expression, abnormal growth, invasion of tumor and disruption of normal functioning which probably results from genomic instability.

The present study was undertaken to identify the feasible and economical method which could be used as a screening test in the population for identifying the effects of genomic instability.

Cancer of the cervix has been the most important cancer in women in India over the past two decades. Cancer of the cervix accounted for 16 per cent of all cancers in women in the urban registries in 2005. However, it constitutes 37 per cent of the cancers in females in Barshi. The highest age specific incidence rate of 98.2 per 100,000 for cancer cervix was seen in the 60-64 yr age group. Since over 70 per cent of the Indian population resides in the rural areas, cancer cervix still constitutes the number one cancer in either sex.

According to Nandakumar et al and Misra et al the highest age specific incidence rate for cancer was seen in more than 60 years of age. Most of the GROUP B cases in our study was above 50 years.

According to Gandhi et al cancer cervix patients were in the age group of 28 to 75 years and they had mostly married young in the age of 15 years. In our study GROUP B cases were in the age group of 21 to 72 years and most of them got married by 18 years. So our findings are well correlated with Gandhi et al , Nandakumar et al and Misra et al.

According to Murthy et al the frequency of cancer cervix showed a progressive risk with increasing parity mostly between Para 2 and 3. But our findings contradict with the findings of Murthy et al and in our study the frequency of cancer cervix is more in Para 1 and 2. According to Castenada et al the relative risk of cervical cancer increases with the more number of abortions. This is similar to the results shown by Gandhi et al where one of the patients with advanced stage cancer cervix had six spontaneous abortions. This is well correlated with our study. In our present study there is linear association between the number of abortions and stage of cancer cervix.

According to Tomatis et al there is a correlation between the low SES and cancer cervix. It has in fact been observed that uneducated women due to lack of knowledge of proper hygiene and preventive measures are more prone to cancer cervix. This

analysis by Tomatis at al and Arora et al are in accordance with our findings.

Data for the MN cells have been grouped with respect to stage of cancer, age of patients and some of the recognized risk factors in cancer cervix etiology like low SES , early marriage and multiparity according to Dutta et al.

Present study confirms the findings reported by Gandhi et al in 2005 where he reports significant damage was observed in patients who were between 21 to 30 years and 51 to 60 years of age. In our study significant MN count was seen age group between 40 to 49 years and more than 50 years of age. But our findings fall in line with the observations made by Leal-Garza et al where the maximum number was seen in the age group between 40 to 60 years of age.

In the present study the frequency of MN cells observed in 51 to 60 years age group are 3 folds higher than in age group of 21 to 30 years. Our findings are in accordance with findings reported by Gandhi et al in 2002. In other intermittent age group there was trend in stepwise increase in MN frequency which was observed in our study too.

Among the GROUP A cases significant MN count was seen in 40 to 49 yrs age group, the reason being the presence of risk factors like illiteracy , low SES , early marriage and more number of conceptions. One patient in GROUP A with significant MN count was a CSW, who's PAP smear showed ASCUS.

For the factor age at marriage significant MN count was seen in patients less than 17 years of age which is in concordance with the findings of Gandhi et al 2003. When the MN count in patients less than 17 years of age was compared to that of patients more than 18 years, non significant result was obtained as in the study done by Gandhi et al in 2002. In the present study MN count was seen in patients married after 18 years in the GROUP A patients.

According to Gandhi et al and Lawson et al the number of pregnancies is significant risk factor for cancer cervix. In the present study the number of conceptions in GROUP A and GROUP B was 20 and 36 respectively. This is in concordance with the observations of Fonn et al in his study.

Kurl et al reported multiparity as a recognized risk factor in cancer cervix. In the present study 46.7% showed less than 2 children and 53.3% more than 3 children which was not statistically

significant. Our findings did not compare well with the findings of Kurl et al. Jain et al also reported similar findings.

In a study by Capalesh et al, he observed that low SES status which has a correlation with poor genital hygiene is a significant risk factor for inducing cancer cervix, which is in accordance with the present study where 73.2% of the cases were in the lower SES. Gandhi et al reported significant difference in MN count in the lower SES and middle SES group. Present study showed in GROUP A, 75% were in the low SES group and 25% in the middle SES group. In GROUP B 73% were in the low SES group and 27% were in the middle SES group.

According to Chakrabarthy et al, there is a strong association between MN frequency and staging of cancer cervix. In our study there is a linear association both MN frequency and stage of the cancer cervix. Mean values of MN was 5 in stage I A, 7 in stage I B, 8.5 in stage II A , 11.8 in stage II B , 15 in stage III A , 17 in stage III B. This linear Association was statistically significant.

The highest frequency of MN observed in stage III B. (0.18 %). She belongs to a low SES and her age at detection was 64 years though her age at marriage was 18 years. Her reproductive history showed 8 conceptions and 4 abortions.

Significant MN count was seen in 4 cases of GROUP A who had normal PAP smear. This is because the sensitivity of MN assay in detecting the genomic instability at an early stage in the carcinogenesis. This has been analysed in the study made by Gandhi et al.

When assessing the mean MN frequency in the GROUP A which was 0.0525% which is in accordance with the study made by Gandhi et al in 2003.

The sensitivity and specificity was calculated for MN assay in urine cytology in the present study in GROUP A. Sensitivity was 83.8% and Specificity was 82.6 %. The Efficiency was found to be 83.3 %. These values showed that MN test in Urothelial cells has got good negative predictive value. This confirms the findings given by Gandhi et al in 2005 where the sensitivity was 72 %, specificity was 83 % and the efficiency was 77.5%.

The emergent views from this study indicate that the MN assay in urothelial cells , after validation, may prove beneficial to perform in screening programmes of cervix cancer given the non-invasive method of sample collection, easier availability of urine and the rapidity of the assay.

SUMMARY AND CONCLUSION

SUMMARY

1. Maximum numbers with abnormal findings on Visual Inspection of Cervix [GROUP B] were in the age group of more than 50 years.

2. 20% of the cases in GROUP B got married before 18 years of age.

3. 72% of the cases in GROUP B had the history of more than 2 abortions.

4. 47% in GROUP B had more than three childbirth.

5. 72.9% of the cases were in the low socioeconomic status group.

6. 50 % of the GROUP cases who were in age group of 40 to 49 yrs and 38.7 % of the GROUP B cases who were more than 50 yrs had significant MN count was more than 50 years of age.

7. A statistically significant MN count was seen in the different stages of cancer cervix.

8. A linear association was noted between the mean MN count and cancer cervix stage.

9. 18.2% of the GROUP A cases had significant MN count.

10. 33.3 % of GROUP (normal findings in Visual Inspection of Cervix) cases who had significant MN count got married before 17 years of age.

11. 6.6% of the total cases were CSWs (commercial sex workers) among them one had erosion cervix and ASCUS on PAP smear. Two of them had significant MN count.

12. Sensitivity and Specificity of MN count in GROUP A was 83.8% and 82.6 % respectively. The Efficiency was 83.3 %.

CONCLUSION

1. Early marriage, recurrent abortions, multiparity are associated with Cytologic changes in cancer cervix.

2. There are cases who had normal findings on Visual Inspection of Cervix but with significant MN count concludes that they are more prone for malignant transformation, needs further follow up.

3. MN assay is an easy, non invasive, cost effective method and hence to be used as a screening test for a large population.

RECOMMENDATIONS

RECOMMENDATIONS

1. Sample size can be taken at a higher scale
2. More number of cells (> 10000) with quantification of DNA damage can be done by automated MN assay and it can be used as screening test.
3. MN assay can also be done in cervical smear cytology.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Boyle P,Ferlay J.Cancer incidence and mortality in Europe, 2004. Annals of Oncology 2005; 16:481-488.

2. Chander S,Chawla S, Rath GK. Screening for cancer of the uterine cervix . Journal of Obstetrics and Gynaecology 1999; 4:651-653.

3. Dutta PK,Upadhya A,Dutta N, Urmil AC. A case control study of cervix cancer patients attending Command hospital, Pune. Indian Journal of Cancer 1990;27:101-108

4. Thompson JD. Cancer cervix .Te Lindes operative Gyanecology.Philadelphia:JB Lippincott Company ; 1992. Pp 1162-1163

5. Misra JS, Srivastava S .Risk factors and strategies for control of carcinoma cervix in India:Hospital based cytological screening experience of 35 years. Indian Journal of Cancer 2009 volume 46 issue 2

6. A Nandakumar, T Ramnath,Meesha Chaturvedi. The Magnitude of cancer cervix in India .Indian Journal of Medical and Research, September 2009, pp219- 221.

7. National Cancer Registry Program. Consolidated report of population based cancer registries 2004 – 2005 .Bangalore: NCRP; 2008.

8. Tomatis,L.Socioeconomic factors and human cancer. International Journal of Cancer 1995, 62 :121-125

9. Shah M,Parikh B,Jani D. Epidemiological study of the cancer of the uterine cervix in adivasi people of five different states of India. Journal of Obstetrics and Gynaecology of India 1985;335:355- 363

10. Murthy VV, Mitra A.B,Das BC,: Chromosomal phenotypes in patients with precancerous lesions of the uterine cervix with progressed to cancer during follow up. Oncology 1988; 45(5):384-388.

11. Murthy NS, Chaudhary K,Saxena C, Trends in cervical cancer in Indian scenario. European Journal of Cancer Prevention 2005; 14:513-518.

12. Mackay HT,Evans ,A: CMDT , Grnaecolgy and Obstetrics, 1997; pp 668-670

13. Richart RM.: Screening: the next century. Cancer, 1995; 76:1919-1927.

14. Arora R;Cervical cancer : A clinical approach. Obstetrics and Gynaecology,1999;4(11):658-666

15. Gandhi G,Sharma P, Kaur A :A comparison of the urothelial cells and cervix scraping in the screening process for cancer of the cervix, Journal of Human Ecology, 2005;17(2):129-135

16. Rengaswamy S,Atul MB: Effective screening programmes for cervical cancer in low and middle income developing countries ,Bulletin of the WHO,2001,79(10)

17. Sankaranaraynan R.Perfomance of Visual inspection after acetic acid application in the detection of cervical cancer precursors, Cancer,1998;83:161-163

18. Murthy NS. Estimation of reduction in life time risk of cervical cancer through one life time screening. Neoplasma, 1993;40:255-258

19. Gandhi ,G,Sharma P,Kaur A :A Comparison of the urothelial cells and cervix scraping techniques in the screening process for cancer cervix, Journal of Human Ecology ,200;17(2):129-135

20. Hodge FS,Harrison S,Gurgin V:Cervical cancer screening. Cancer ,1998;83:1799-1804

21. Pawitan JA.Biological tests for Mutagenesis in man. A clinical review. Medical Progress , 16-18 July 1999

22. Genetic toxicity .Available fromhttp://www.cerep.fr/cerep/users/pages/download/documents/marketing/pharmacology/application/genetoxicity .pdf. January 2007

23. Fenech M. Chromosomal biomarkers of genomic instability relevant to cancer .DDT 2002;7(22):1128-1137

24. Ahuja YR, Rajeswari ,N, Anuradha G : Molecular Epidemiology of individual risk to cancer. 1996;165-170.

25. Stitch HF,Curtis JR, Rosin M: Micronuclei in exfoliated cells as a tool for studies in cancer risk and cancer intervention. Cancer letter , 1984; 22:241-253

26. Decordier I,Kirsch V:The in vitro micronucleus test :from past to future. Mutation and Research.2006;607(1):2-4

27. Garewal HS,Ramsey L,KaugersG.Clinical experience with micronucleus assay.Journal of cellular Biochemistry, 2004;53(17):206-212.

28. Martino Roth MG,Viegas J,Roth DM. Occupational genotoxicity risk evaluation through comet assay and the micronucleus test. Genetics and Molecular Research 2003;2(4):410-417

29. Burgaz S,Erdem O,Cakmak G. Cytogenetic analysis of buccal cells from shoe workers and pathology and anatomy from lab workers exposed to n hexane toluene and methyl ketone. Biomarkers 2002;7(2): 151-161

30. Fenech M,Holland N,Chang WP, Zeiger E, Bonassi S. The Human micronucleus project – An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans. Mutation and Research 1999;428:271-283

31. Kamboj M,Mahajan S. Clinical oral investigations 2007;11(2):121-126

32. Halder A, Chakraborthy T,Mandal K Gure PK. Comparative study of exfoliated oral mucosal cell micronuclei frequency in normal precancerous and malignant epithelium. International Journal of Human Genetics 2004;4(4):257-260

33. Yadav JS , Chadha P. Genotoxic studies in pan masala chewers: A high cancer risk group. International Journal of Human genetics 2002;2(2):107-112

34. Lizia MF,Dias FL,Antunes MG. Prevalence of micronuclei in exfoliated urine cervical cells from patients with risk factors for cervical cancer. Sao Paulo medical Journal. 2008;126(6):323-328

35. Leal Garza, Cerda Flores.Micronuclei in cervical smears and peripheral blood lymphocytes from women with and without cervical cancer. Mutation and Research.2002;515(1-2): 57-62

36. Gandhi G,Sharma P,Kaur,Badaruddozza. The micronucleus test in urothelial cells and uterine smears of cancer cervix patients: A Comparison. International Journal of Human Genetics,2003;3(2):121-126.

37. Gursatej Gandhi ,Pankaj Sharma. The micronucleus test in urothelial cells in cancer cervix patients. Indian Journal of Human Genetics.2002; 8 (2) :69-72

38. Yildrim IH, Yesilada E,Yologulu S. Micronucleus frequency in peripheral blood an exfoliated mucosal cells of of untreated cancer patients. Genetika 2006;42(5):705-71.

39. G. Casartelli, S. Monteghirfo, M. Deferrari, S. Bonatti, M.Scala, S. Toma, G. Margarino, A. Abbondandolo, Staining of MN in squamous epithelial cells of human oral mucosa, Annals of. Quantitative Cytology and Histology, 1999;19:475–481.

40. Belin M.P. Copper, B.J.M. Braakhuis, G.B. Snow,J.P.A. Baak, Standardization of counting micronuclei: definition of a protocol to measure genotoxic damage in human exfoliated cells, Carcinogenesis,1995;16:2395–2400.

41. Fenech M , Bonassi S, Lando C,Yi Ping Lin. Human Micronucleus Project:International database comparisons for resulst with cytokinesis block micronucleus assay in human lymphocytes : Effect of laboratory protocol,scoring criteria and host factors on the frequency of Micronuclei. Environmental and Molecular Mutagenesis,2001;37:31-45.

42. Walton RJ.Editorial: The task force on cancer cervix screening programs. Can Med Assoc J 1976; 114:981.

43. Nanda K,McCrory DC,Myers et al .Accuracy of the Papanicolaou test in screening for and follow up of cervical cytologic abnormalities: a systemic review. Ann Intern Med 2000; 132:810-819.

ANNEXURES

ANNEXURE – I

Points for identification of MN:

1. Morphologically identical but smaller than nuclei
2. Round or oval in shape
3. Diameter between 1/3rd and 1/16th of main nuclei
4. Nonrefractile
5. Not linked to main nucleus
6. May touch but not overlap the main nucleus and the micronuclear boundary should be distinguishable from the nuclear boundary.
7. Same color as nucleus
8. Similar staining intensity or more as nucleus

ANNEXURE – II

PROFORMA

Name Age	Religion Occupation	Address Phone no-	Op no: Urine sample no:
Complaints		Duration	
Menarche Menopause Postmenopausal bleeding		Menstrual history LMP	
Marital history		Obstetric history	
No of children		Alive Last childbirth	
No of abortions		Past history	
Contraception		Other systems	
Examination of Abdomen Visual inspection of cervix		Diagnosis Results	

Observation

Slide no:	Op no:	Urine sample no:	Visual inspection of cervix	Diagnosis	Results		
					No of cells	MN	MN frequency